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Applicant:	CHABOT, et al.	Examiner:	Unknown
Serial No.:	09/832,183	Group Art Unit:	1615
Filed:	April 10, 2001	Docket No.:	9555.115US01
Title:	METHOD FOR MODULATING SPLICING AND/OR ALTERNATIVE SPLICING, AND FOR IDENTIFYING ALTERNATIVELY SPLICED UNITS IN GENES		

CERTIFICATE UNDER 37 CFR 1.10

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By: 
Name: Omesh Singh

SUBMISSION OF PRIORITY DOCUMENTS

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

Applicants enclose herewith one certified copy of a Canadian application, Serial No. 2,305,956, filed April 10, 2000, the right of priority of which is claimed under 35 U.S.C. Section 119.

Respectfully submitted,

MERCHANT & GOULD, P.C.
P.O. Box 2903
Minneapolis, Minnesota 55402-0903
(612) 332-5300

Dated: July 27, 2001
By 
Gregory A. Sebald
Reg. No. 33,280

GAS/sef

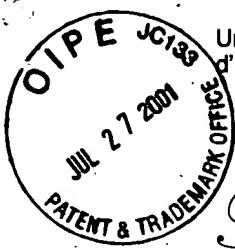


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Specification and Drawings, as originally filed, with Application for Patent Serial No:
2,305,956, on April 10, 2000, by UNIVERSITÉ DE SHERBROOKE, assignee of Benoît
Chabot and Lucie Bolduc, for "Agents which Modulate the Function of hnRNP A1
Thereby Affecting Alternative Splicing and/or Telomere Biogenesis, and Method of
Identifying Same".

J. G. Négoce
Agent certificateur/Certifying Officer

May 24, 2001

Date

Canada

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O P I C C I P O

ABSTRACT OF THE DISCLOSURE

The present invention relates to alternative RNA splicing which is involved in the production of protein isoforms with distinct 5 activities. The present invention also relates to telomeres and to their effect on proliferation and senescence in cells. The present invention concerns the ability of DMSO to affect the activity of hnRNP A1, a protein that controls alternative splicing and telomere length.

TITLE OF THE INVENTION

AGENTS WHICH MODULATE THE FUNCTION OF hnRNP A1 THEREBY AFFECTING ALTERNATIVE SPLICING AND/OR TELOMERE BIOGENESIS, AND METHODS OF IDENTIFYING SAME

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FIELD OF THE INVENTION

The present invention relates to alternative RNA splicing which is involved in the production of protein isoforms with distinct activities. The present invention also relates to telomeres and to their effect on proliferation and senescence in cells. The present invention concerns the ability of DMSO to affect the activity of hnRNP A1, a protein that controls alternative splicing and telomere length.

BACKGROUND OF THE INVENTION

15 Dimethyl sulfoxide (DMSO) is often used to promote cell differentiation of tumor cell lines. For example, the treatment of mouse erythroleukemic cells and mouse neuroblastoma cells with 2% DMSO induces morphological changes and phenotypic differentiation into red blood cells and neurons, respectively. DMSO also promotes
20 differentiation of rhabdomyosarcoma cells in vitro (Prados et al. 1993, Cell Mol Biol. 39:525), induces the differentiation and apoptosis of the human U937 monoblast leukemia cell line into monocyte/macrophage (Nicholson et al. 1992, J. Biol. Chem. 267:17849; Chateau et al. 1996, Anal. Cell Pathol. 10:75), and stimulates the differentiation of a human ovarian
25 adenocarcinoma cell line (Grunt et al. 1992, J. Cell. Sci. 103:501). DMSO is also used to promote the differentiation of hepatocytes in culture

(Kojima et al. 1997, *Hepatology* 26:585). Contrastingly, DMSO can be used to prevent terminal differentiation of myoblasts (Blau and Epstein 1979, *Cell* 17:95), to inhibit adipocytes differentiation (Wang and Scott 1993, *Cell Prolif.* 26:55), to block the differentiation of antibody-producing plasma cells (Teraoka et al. 1996, *Exp. Cell Res.* 222:218), and to interfere with the differentiation of chick embryo chondrocytes (Manduca et al. 1988, *Dev. Biol.* 125:234). More recently, DMSO treatment has been used to either induce apoptosis (or programmed cell-death) in a pre-T cell line (Trubiani et al. 1999, *Cell Prolif.* 32:119), or, in contrast, to inhibit cell density-dependent apoptosis (Fiore and Degrassi 1999, *Exp. Cell Res.* 251:102). Thus, depending on the cell line used, DMSO can either promote differentiation and apoptosis, or block differentiation and apoptosis. The use of DMSO in pharmaceutical formulations is known, for example, from U.S.P. 4,296,104; 4,652,557; and 5,516,526.

The mechanism by which these events occurs are unclear. Because DMSO is used to facilitate DNA uptake during transfection procedures (e.g., Melkonyan et al. 1996, *Nucl. Acids. Res.* 24:4356), DMSO has been proposed to affect cell membrane and signal transduction. Consistent with this view DMSO treatment can affect the expression of protein kinase C (Makowske et al. 1988, *J. Biol. Chem.* 263:3402). DMSO treatment also promotes changes in the abundance of certain mRNAs and spliced isoforms (Tam et al. 1997, *J. Lipid Res.* 38:2090; Srinivas et al. 1991, *Exp. Cell Res.* 196:279; Bahler and Lord 1985, *J. Immunol.* 134:2790; Campbell et al. 1990, *Genes Dev.* 4:1252). These changes may be mediated by an indirect effect of DMSO on transcription and alternative splicing, for example a direct effect on signal

transduction (a kinase or phosphatase) may have consequences on transcription and/or alternative RNA splicing. Among the genes reported to be affected in their alternative splicing is the NCAM pre-mRNA. A 2% DMSO treatment of N2a cells promotes an increase in the inclusion of 5 neuro-specific exon 18 (Pollerberg et al. 1985, J. Cell. Biol. 101: 1921; Prentice et al. 1987, EMBO J. 6: 1859). Genes whose alternative splicing profiles have been reported to be affected by treatment with DMSO include the amyloid precursor protein (Pan et al. 1993, Brain Res Mol Brain Res. 18:259), the serotonin 5-HT3 receptor-A mRNA (Emerit et al. 10 1995, J. Neurochem. 65:1917), p53 (Bendori, 1987, Virology 161:607). DMSO has also been associated to affect c-myc mRNA elongation and maturation (Eick 1990, Nucl. Acids Res. 18:1199) and the mRNA translation of other genes (Yenofsky et al., 1983, Mol. Cell. Biol. 3:1197). A survey of the scientific literature reveals that the mechanism of action 15 of DMSO, as far as gene expression is concerned, has not been seriously investigated.

It is shown herein that DMSO can control alternative RNA splicing directly. This direct link is based on the fact that DMSO affects the alternative splicing of pre-mRNAs when assayed in extracts 20 prepared from human HeLa cells (i.e., an in vitro splicing system). Thus, the effects observed must affect factors involved in alternative splicing because the effects seen cannot be occurring through membrane-mediated effects, transcription, translation, etc....

DMSO does not affect constitutive (generic) splicing in 25 nuclear extracts. A pre-mRNA which has been used as model to study

constitutive splicing remains spliced efficiently in the presence of up to 2.5% DMSO.

However, DMSO can completely abrogate distal 5' splice site utilization when using a model pre-mRNA that has been used 5 previously to show that the binding of hnRNP A1 to high-affinity A1 binding sites can promote distal 5' splice site selection (Blanchette and Chabot 1999, EMBO J. 18: 1939). When distal 5' splice site selection is promoted by an hnRNP A1-independent mechanism (e.g., secondary structure formation), 5' splice site selection is not affected by DMSO. The 10 effect of DMSO is reversible since the addition of recombinant A1 protein in the DMSO-containing extract restored efficient distal 5' splice site selection. This effect is also specific since the addition of equivalent amounts of GST or gene 32 protein has no effect when added to DMSO-containing extracts.

15 DMSO also affects 3' splice site selection. Using model pre-mRNA that are efficiently spliced to the distal 3' splice site in an hnRNP A1-dependent manner, DMSO can neutralize this activity. However, when the distal 3' splice site is selected preferentially because 20 a silencer element represses the proximal 3' splice site, then DMSO has no effect.

Given that DMSO does not prevent the binding of A1 to high-affinity binding sites, the instant results suggest that DMSO prevents interactions between hnRNP A1 proteins. This interaction was proposed to occur in Blanchette and Chabot 1999 (supra) and is shown 25 in Figure 8.

It is shown that DMSO affects NCAM splice site selection in vitro using a model pre-mRNA carrying a NCAM 3' splice site. It was already known that the treatment of N2a cells with DMSO promoted inclusion of NCAM exon 18. These results suggest that DMSO 5 affects cell differentiation through direct modulation of alternative splicing.

ALTERNATIVE SPLICING

Importance of alternative splicing

Alternative splicing is a process which involves the 10 selective use of splice sites on a mRNA precursor. Alternative splicing allows to produce many proteins from a single gene and therefore allows to generate proteins with distinct functions. Alternative splicing events can occur through a variety of ways including exon skipping, the use of mutually exclusive exons and the differential selection of 5' and/or 3' 15 splice sites. For many genes (e.g., homeogenes, oncogenes, neuropeptides, extracellular matrix proteins, muscle contractile proteins), alternative splicing is regulated in a developmental or tissue-specific fashion. Alternative splicing therefore plays an important role in gene expression. It is estimated that 5-10% (10 000) of all of our genes are 20 alternatively spliced. The number of isoforms made from alternative splicing of a single gene vary from 2 to more than a 1000. Assuming an average of five isoforms per gene that is alternative spliced, alternative splicing would be responsible for the production of 50 000 different proteins.

25 Alternative splicing of mRNA precursors plays an important role in the regulation of mammalian gene expression. The

regulation of alternative splicing occurs in cells of various lineages and is part of the expression program of a large number of genes. Recently, it has become clear that alternative splicing controls the production of proteins isoforms which, sometimes, have completely different functions.

5 Oncogene and proto-oncogene protein isoforms with different and sometimes antagonistic properties on cell transformation are produced via alternative splicing. Examples of this kind are found in Makela et al. 1992, Science 256:373; Yen, et al. 1991, Proc.Natl.Acad.Sci.U.S.A. 88:5077; Mumberg et al. 1991, Genes.Dev. 5:1212; Foulkes, N.S. and Sassone-
10 Corsi, P. 1992, Cell 68:411.

Despite the biological relevance of alternative splicing to cell growth, cell differentiation and mammalian development, a detailed understanding of the process is still lacking. In most cases, the nature of regulatory elements, the identity of *trans*-acting factors and the 15 mechanisms involved in the regulation remain unknown. A lot of efforts is now devoted to understand how splicing regulation is achieved in mammalian cells (Chabot 1996, Trends Genet.). There thus remains a need to better understand the relevance of alternative splicing to cell growth, cell differentiation and mammalian development. There also 20 remains a need to better understand how splicing and particularly alternative splicing and the regulation thereof are controlled in cells. In addition, there remains a need to identify agents which can modulate alternative splicing in cells and animals.

Several splicing regulators affect the initial ATP-
25 independent steps of spliceosome assembly, which include the recognition of the 5' splic site by U1 snRNP and the recognition of the

polypyrimidine tract/3' splice site by U2AF⁶⁵. The best characterized mammalian regulators are SR proteins, a family of splicing factors which contain arginine-serine-rich sequences (reviewed in Fu, 1995; Manley and Tacke, 1996; Chabot, 1996). SR proteins generally favor proximal (internal) 5' splice site or 3' splice site selection *in vitro* and exon inclusion *in vivo* (Fu et al., 1992; Mayeda et al., 1993; Cáceres et al., 1994; Zahler et al., 1995). One member of this family of proteins (SF2/ASF) promotes U1 snRNP binding to weak 5' splice sites, and favors U1 snRNP binding to all 5' splice sites in more complex pre-mRNAs (Eperon et al., 1993; 5 Kothz et al., 1994). Thus, increasing the proportion of pre-mRNA molecules bound by U1 at all competing 5' splice sites should favor the use of the internal site because of its closer proximity to the 3' splice site. In addition, U2AF⁶⁵ binding to weak 3' splice sites is stimulated by SR 10 proteins bound to a downstream splicing enhancer (e.g., purine-rich sequence) (Lavigueur et al., 1993; Sun et al., 1993; Staknis and Reed, 1994; Wang et al., 1995). Because SR proteins can interact simultaneously with U2AF and the U1 snRNP 70K protein (Wu and Maniatis, 1993), SR proteins are thought to participate in the stimulation 15 of U2AF binding through exon-bridging interactions with a downstream U1-bound 5' splice site (Wang et al., 1995; reviewed in Chabot, 1996). Current results also suggest that SR proteins promote commitment between a pair of splice site by favoring an intron-bridging interaction 20 between U1 snRNP and U2AF (Fu et al., 1993; Wu and Maniatis, 1993; Staknis and Reed, 1994; Wang et al., 1995). While SR proteins can stimulate each of the initial ATP-independent step of spliceosome 25 assembly, in some cases SR proteins may act as splicing repressors,

either by binding to sites that sterically occlude spliceosome assembly (Kanopka et al., 1996), or by blocking the binding of more active SR proteins (Gallego et al., 1997). The current understanding of the role of SR proteins is still rudimentary and more work is needed to understand 5 the biological function of each member, and the role of phosphorylation by specific kinases that modulate their localization and activity in the nucleus (Gui et al., 1994; Colwill et al., 1996; Xiao and Manley, 1997). There thus remains a need to better understand the role of SR proteins and of phosphorylation by specific kinases in alternative splicing.

10 The negative regulation of U1 snRNP and U2AF⁶⁵ binding is also a strategy used to modulate splice site selection and often requires the participation of hnRNP or related proteins. In the *Drosophila* somatic P-element pre-mRNA, the formation of an RNA-protein complex containing U1 snRNP, the soma-specific PSI protein and the ubiquitous 15 hrp48 protein prevents downstream 5' splice site recognition (Siebel et al., 1995). HnRNP I, also called PTB, has been implicated in the regulation of several alternatively spliced genes, including α - and β -tropomyosin, c-*src* and GABA_A receptor γ 2 subunit, possibly by interfering with the adjacent binding of U2AF (Lin and Patton, 1997; reviewed in Valcarcel 20 and Gebauer, 1995). The hnRNP F protein has been found to be part of a complex involved in activating neural-specific splicing of the alternative c-*src* exon N1 (Min et al., 1995). The more abundant members of the family of hnRNP proteins (A and B group) can antagonize the effect of SR 25 proteins on splice site selection (Mayeda and Krainer, 1992; Mayeda et al., 1993; Yang et al., 1994).

The heterogeneous nuclear ribonucleoparticle (hnRNP) protein A1 is one of the most abundant nuclear protein in actively growing mammalian cells. The hnRNP A1 pre-mRNA is itself alternatively spliced to yield the A1 and A1b proteins which differ in their ability to affect splice site selection. HnRNP A1 affects 5' splice site selection through the presence of high-affinity A1 binding sites (Blanchette and Chabot 1999, *supra*). The mechanism of action that was proposed to explain the function of A1 is as follows: A1 proteins bound to high-affinity A1 binding sites would interact with one another through their glycine-rich domain.

5 This interaction could change the conformation of the pre-mRNA and increase the frequency of commitment between the distal 5' splice site and the distal 3' splice site (see Fig. 6A in Blanchette and Chabot, 1999, *supra*).

10 This interaction could change the conformation of the pre-mRNA and increase the frequency of commitment between the distal 5' splice site and the distal 3' splice site (see Fig. 6A in Blanchette and Chabot, 1999, *supra*).

15 The presence and distribution of A1 binding sites has been examined in a subset of pre-mRNAs that are alternatively spliced (Chabot et al. 1997, *Mol. Cell. Biol.* 17: 1776; Blanchette and Chabot 1999, *supra*).

TELOMERE BIOGENESIS

20 Importance of telomeres

Telomeres are the DNA structure at the ends of the chromosomes of eukaryotes, including human, and are comprised of variable lengths of double-stranded repeats terminating with single-stranded G-rich repeats originally identified in yeast and protozoa

25 (Makarov et al., 1997, *Cell* 88:657-666; McElligott and Wellinger 1997, *EMBO J.* 16:3705).

Review articles concerning telomeres include Greider, 1996, Ann. Rev. Biochem. 65:337 and Zakian, 1995, Science 270:1601. Relevant articles on various aspects of telomeres include Cooke and Smith, 1986, Cold Spring Harbor Symp. Quant. Biol. 51:213; Morin, 1989, 5 Cell 59:521; Blackburn et al., 1989, Genome 31:553; Szostak, 1989, Nature (London) 337:303; Gall, 1990, Nature (London) 344:108; Henderson et al., 1990, Biochemistry 29:732; Gottschling et al., 1990, Cell 63:751; Harrington et al., 1991, Nature (London) 353:451; Muller et al., 1991, Cell 67:815; Yu et al., 1991, Cell 67:823; Gray et al., 1991, Cell 10 67:807; de Lange, 1995, "Telomere Dynamics and Genome Instability in Human Cancer", E. Blackburn and C. W. Greider (eds), in Telomeres, Cold Spring Harbor Laboratory Press, pp. 265-293; Rhyu, 1995, J. Natl. Cancer Inst. 87:884; Greider and Harley, 1996, "Telomeres and Telomerase in Cell Senescence and Immortalization", in Cellular Aging and Cell Death, Wiley-Liss, Inc., pp. 123-138. Other articles of some 15 relevance include Lundblad et al., 1989, Cell 57:633 and Yu et al., 1990, Nature (London) 344:126. Thus, telomeres are involved in the maintenance of chromosome structure and function. Furthermore, it appears that loss of telomeric DNA activates cellular processes involved 20 in the detection and control of DNA damage, and affects cellular proliferation and senescence.

Maintenance of the integrity of telomeres is essential for cell survival (Muller, 1938, The Collecting Net 13:181-195; Sandell et al., 1993, Cell 75:729-739). The proliferative potential of cells has been 25 correlated with alterations in the length of these tandemly repeated sequences (Zakian, 1989, Ann. Rev. Genet. 23:579-604; Counter et al.,

1992, EMBO J. 11:1921-1929). In addition, maintenance of telomere length and the regulation thereof are essential, pluripotent cellular functions as they are involved in the transmission of genetic information to daughter cells, senescence, cell growth and cancer (Harley, 1991,
5 Mutation Research 256:271-282; and Blackburn, 1992, Annu. Rev. Biochem. 61:113-129).

The finite replicative capacity of normal human cells, e.g., fibroblasts, is characterized by a cessation of proliferation in spite of the presence of serum growth factors. This cessation of replication after
10 a maximum of 50 to 100 population doublings in vitro is referred to as cellular senescence. See, Goldstein, 1990, Science 249:1129; Hayflick and Moorehead, 1961, Exp. Cell Res. 25:585; Hayflick, 1985, *ibid.*, 37:614; Ohno, 1979, Mech. Aging. Dev. 11:179; Ham and McKeehan, 1979, "Media and Growth Requirements", W. B. Jacoby and I. M. Pastan
15 (eds), in Methods of Enzymology, Academic Press, NY, 58:44-93. The replicative life span of cells is inversely proportional to the in vivo age of the donor (Martin et al., 1979, Lab. Invest. 23:86; Goldstein et al. 1969, Proc. Natl. Acad. Sci. USA 64:155; Schneider and Mitsui, 1976, *ibid.*, 73:3584) and is therefore suggested to reflect in vivo ageing on a cellular
20 level.

Cellular immortalization (unlimited life span) may be thought of as an abnormal escape from cellular senescence (Shay et al., 1991, Exp. Cell Res. 196:33). Normal human somatic cells appear to be mortal, i.e., have finite replication potential. In contrast, the germ line and
25 malignant tumor cells are immortal (have indefinite proliferative potential). Human cells cultures in vitro appear to require the aid of transforming

oncoproteins to become immortal and even then the frequency of immortalization is 10^{-6} to 10^{-7} (Shay et al., 1989, *Exp. Cell Res.* 184:109). A variety of hypotheses have been advanced over the years to explain the causes of cellular senescence. One such hypothesis proposes that 5 the loss of telomeric DNA with age, eventually triggers cell cycle exit and cellular senescence (Zakian, 1989, *Ann. Rev. Genet.* 23:579-604; Harley et al. 1990, *Nature (London)* 345:458-460; Hastie et al., 1990, *Nature (London)* 346:866-868; Allsopp et al., 1992, *Proc. Natl. Acad. Sci. USA* 89:10114-10118; Counter et al., 1992, *EMBO J.* 11:1921-1929).

10 Human primary fibroblasts in culture enter crisis after a precise number of cell division associated with gradual telomere shortening, at which point all the cells die (de Lange, 1994, *Proc. Natl. Acad. Sci. USA* 91:2882-2885). Mouse primary fibroblasts have longer and/or more stable telomeres and display a similar behavior when 15 cultured in vitro (Prowse and Greider, 1994, *Proc. Natl. Acad. Sci. USA*, 92:4818-4822). However, after crisis, primary mouse cells in culture spontaneously immortalize with a frequency of 10^{-6} , possibly because longer telomeres facilitate the growth of mutant cells (de Lange, 1994, *Proc. Natl. Acad. Sci. USA* 91:2882-2885).

20 It should be noted, as mentioned above, that other hypotheses have been advanced to explain senescence and that there is yet to be a consensus or a universally accepted hypothesis therefor. Previously, the causal relationship between telomeres and cancer/ageing/senescence had been built entirely on correlative studies.

25 Recent data has shown that telomeres play a direct role in cell senescence and transformation. Indeed, Wright et al., 1996, *EMBO*

J. 15:1734-1741, using telomerase-negative cells which have limited life span in tissue culture, have shown that the introduction of oligonucleotides carrying telomeric repeats causes telomere elongation and increases the proliferative capacity of these cells. Moreover, the 5 authors state that "previous studies had shown a remarkable correlation between telomere length and cellular senescence. The present results provide the first experimental evidence for a true causal relationship between telomere length and a limited proliferative capacity". Feng et al., 1995, Science 269:1236-1241 showed that human cell line (HeLa) 10 transfected with an antisense telomerase RNA, loose telomeric DNA and begin to die after 23-26 cell doublings. The authors claim that "the results support the hypothesis that telomere loss leads to crisis and cell death once telomeres are shortened to a critical length". Telomerase is a multi-component ribonucleoprotein complex. The RNA component of the 15 human telomerase has been identified. The catalytic protein subunit has recently been cloned (Nakamura et al. 1997, Science 277:955; Meyerson et al. 1997, Cell 90:785; Harrington et al. 1997, Genes Dev. 11:3109).

More recent advances have confirmed the role of 20 telomeres in cell senescence. Overexpression of the catalytic protein component of telomerase can lead to telomere elongation and extension of the proliferative capacity of telomerase-negative fibroblasts in culture (Bodnar et al. 1998, Science 279:349; Vaziri and Benchimol 1998, Curr. Biol. 8:279). Overexpression of this protein also prevents the accelerated ageing of human fibroblasts derived from patients with Werner syndrome 25 (Wyllie et al. 2000, Nat. Genet. 24:16). Mice and murine ES cells that do not express telomerase RNA show telomere shortening and become

impaired in long-term viability (Lee et al. 1998, *Nature* 392:569; Niida et al. 1998, *Nat. Genet.* 19:203). Recent studies have also supported the role of telomeres in cellular transformation. The expression of a catalytically inactive form of telomerase or the inactivation of telomerase 5 RNA in human immortal and cancer cell lines promotes telomere shortening, growth arrest and cell death (Hahn et al. 1999, *Nat. Med.* 5:1164; Herbert et al. 1999, *Proc. Natl. Acad. Sci. USA* 96:14276; Zhang et al. 1999, *Genes Dev.* 13:2388).

10 The length of telomeres and cell viability can also be affected by proteins that binds to vertebrate telomeres. TRF1 and TRF2 are proteins that bind to double-stranded telomeric repeats. Overexpression of TRF1 promotes telomere shortening (van Steensel and de Lange 1997, *Nature* 385:740). Expression of a dominant negative version of TRF2 promotes end-to-end fusion of chromosomes, an event 15 which leads to p53-dependent cell death by apoptosis (van Steensel et al. 1998, *Cell* 92:401; Karlseder et al. 1999, *Science* 283:1321).

20 The postulated link between senescence/proliferation of cells and telomere length has led to therapeutic and diagnostic methods relating to telomere length or to telomerase, the ribonucleoprotein enzyme involved in the synthesis of telomeric DNA. PCT Publication No. 93/23572 describes oligonucleotide agents that either reduce the loss of telomeric sequence during passage of cells in vitro, or increase telomeric length of immortal cells in vitro. The same type of approach is also taught in PCT Publication No. 94/13383 and U.S. Patent 5,484,508 which refer 25 to methods and compositions for the determination of telomere length and telomerase activity, as well as to methods to inhibit telomerase activity in

the treatment of proliferative diseases. Methods to increase or decrease the length of telomeres through an action on telomerase is also taught. The agents which are shown to reduce telomere loss of telomere length during proliferation are oligonucleotides which promote synthesis of DNA at the telomere ends, as well as telomerase.

5 PCT Publication No. 95/13383 discloses a method and compositions for increasing telomeric length in normal cells so as to increase the proliferative capacity of the cells and to delay the onset of cellular senescence. PCT Publication No. 96/10035 teaches that telomere length serves as a biomarker for cell turnover. Furthermore, it discloses 10 that measurement of telomere length can be used to diagnose and stage cancer and other diseases as well as cell senescence.

15 PCT publication WO98/11204 teaches two nucleic acid sequences termed TPC2 and TPC3 and amino acid sequences of the polypeptides encoded thereby which can be used to detect regulators of telomere length and telomerase activity in mammalian cells. TPC3 is shown to regulate telomerase activity and telomere length.

20 PCT publication WO98/11207 teaches telomerase reporter constructs to be used in assessing the transcriptional activity of mammalian telomerase gene transcription regulatory region. This application also relates to the use of these constructs to identify agents which modulate transcription of the telomerase gene.

25 Proteins that bind mammalian telomeric repeats, either to double-stranded repeats or single-stranded repeats, are also targets for telomere length regulation.

U.S. Patent 5,733,730 and PCT WO97/08314 relate to the double-stranded DNA binding factor TRF and discloses a method of purifying telomerase from mammalian cells.

PCT Publication No. WO 98/00537 relates to the single-stranded DNA binding factor A1/UP1 and discloses methods and compositions to increase or decrease telomeric length. A1 is a member of the abundant family of heterogeneous nuclear ribonucleoprotein particles (hnRNP) proteins (Dreyfuss et al. 1993. Ann. Rev. Biochem. 62:289). There are over 20 such hnRNP proteins in human cells. 5 HnRNP A1 can modulate telomere length once introduced into a mouse cell line (WO98/00537; and La Branche et al., 1998. Nat. Genet. 19:199-202). UP1 lacks the last N-terminal 124 aa of A1 (the glycine-rich domain), but shares with A1 the first 196aa. The first 196aa comprises 10 two RNA Recognition Motifs (RRMs); RRM1 extending from aa 15-93, and RRM2 extending from aa 106-184. UP1 can modulate telomere length once introduced into a mouse or a human cell line (WO98/00537; and La Branche et al., 1998., supra). 15

A separate embodiment describes the ability of hnRNP A1 and UP1 to promote telomere elongation in mammalian cells 20 (WO98/00537; and La Branche et al., 1998. Nat. Genet. 19:199- 202). Also described is the ability of hnRNP A1 and UP1 to bind with high affinity and with specificity single-stranded vertebrate telomeric DNA sequences. Moreover, UP1 can recover telomerase from a cell extract. It has been shown in a separate embodiment (submitted) that both A1 25 and UP1 also interact directly and with specificity with the 5' end of human telomerase RNA (hTR).

Based on these observations, the interaction of A1/UP1 to telomeric DNA or telomerase RNA represent valid targets to affect telomere structure. Preventing the binding of A1 to telomeric DNA or telomerase RNA in transformed cells should compromise the integrity of 5 telomeres and lead to cell death. Likewise, preventing the interaction of A1/UP1 with other proteins (other telomeric factors) should also compromise the integrity of telomeres and lead to cell death.

In a different embodiment (submitted and see Dallaire et al. 2000, *J. Biol. Chem.* in press), it is also shown that the binding of 10 A1/UP1 to telomeric sequences protects these sequences from nuclease attack (endonuclease and exonucleases). Thus, interfering with A1/UP1 binding or the interaction between A1/UP1 molecules would interfere with the protective capacity associated with A1/UP1 binding and may lead to telomere erosion. Alternatively, preventing A1/UP1 binding may produce 15 telomeric ends that are recognized as double-stranded breaks, an event that would lead to rapid cell growth arrest.

The ability of UP1 to recover telomerase activity, and the ability of A1/UP1 to interact with human telomerase RNA suggest that another way by which A1/UP1 may control the length of telomeres is by 20 recruiting telomerase to the ends of chromosomes. Thus, interfering with the binding of A1/UP1 to telomerase RNA or interfering with the interaction of A1/UP1 with other telomerase proteins would interfere with telomere maintenance and should promote telomere shortening, similar to the effects seen when the activity of telomerase is targeted.

25 There remains a need to identify agents that will enable a modulation of telomere length and/or telomere replication.

The present invention seeks to meet these and other needs.

The present description refers to a number of documents, the content of which is herein incorporated by reference in their entirety.

SUMMARY OF THE INVENTION

It is therefore an aim of the present invention to overcome the drawbacks in the prior art and enable a better 10 understanding of the relevance of alternative splicing to cell growth, cell differentiation and mammalian development.

It is also an aim of the present invention to better understand how splicing and the regulation thereof are controlled in cells and to provide agents which modulate alternative splicing in cells and 15 animals.

In one particular embodiment, the present invention seeks to provide agents which can modulate alternative splicing and telomere biogenesis through a modulation of hnRNP A1 function. In an especially preferred embodiment of the present invention, DMSO is used 20 to control alternative splicing and/or telomere biogenesis by modulation of splicing-dependent on hnRNP A1.

In another embodiment of the present invention, there is provided methods to identify pre-mRNAs whose alternative splicing profile is dependent on hnRNP A1. In a particular embodiment, such a 25 method comprises an incubation of a cell or extract with DMSO and a monitoring of the splicing profile of at least one gene in such cell or

extract using known assays such as RT-PCR. In another embodiment of the present invention, a confirmation of the role of hnRNP A1 in splicing can be obtained using *in vitro* assays such as, for example, a splicing assay.

5 The present invention also provides methods and assays to identify agents which can modulate alternative splicing and/or telomere biogenesis. In one particular embodiment of the present invention, such a method comprises an incubation with a splicing extract which contains pre-mRNA, wherein change in splicing of this pre-mRNA
10 can be detected and/or measured, and comparing qualitatively or quantitatively the splicing of this pre-mRNA in the presence of an agent (or a library thereof) and in the absence of this agent (or library thereof), wherein an agent which can modulate splicing and/or telomere biogenesis is identified when qualitative or quantitative difference in the
15 spliced products or splicing intermediates is measurably different in the presence of the agent as compared to in the absence thereof.

For the purpose of the present invention, the following abbreviations and terms are defined below.

20 The terminology "A1" relates to hnRNPA1 having an activity in telomere biogenesis and alternative splicing when in their native form. It will be clear to the skilled artisan that recombinants, derivatives or portions of A1 can also be used and tested in accordance with the present invention.

25 By "increased rate of proliferation" of a cell, it is meant that a cell has a higher rate of cell divisions compared to normal cells of that cell type, or compared to normal cells within other individuals of that

cell type. Examples of such cells but not limited to these, include the CD4⁺ cells of HIV-infected individuals, connective tissue fibroblasts associated with degenerative joint diseases, age-related muscular degeneration, astrocytes associated with Alzheimer's Disease and 5 endothelial cells associated with atherosclerosis. In each case, one particular type of cell or a group of cells is found to be replicating at an increased level compared to surrounding cells in those tissues, or compared to normal individuals, e.g., individuals not infected with the HIV virus. Thus, the invention features administering to those cells an agent 10 which reduces the loss of telomere length in those cells while they proliferate. The agent itself need not slow the proliferation process, but rather allow the proliferation process to continue for more cell divisions than would be observed in the absence of the agent. The agent may also be useful to slow telomere repeat loss occurring during normal aging, and 15 for reducing telomere repeat loss while expanding cell number *ex vivo* for cell-based therapies. The agent could thus simply stabilize telomere length.

The assessment of the effect of agents on telomere length modulation or on telomere replication can be assessed by 20 analyzing their effect in modulating the length of telomeres. For example, a particular cell having a known telomere length is chosen and allowed to proliferate and the length of telomere is measured during proliferation. Analysis of telomere length in cells expressing different derivatives or fragments can be identified using methods described below 25 or other methods known to a person of ordinary skill. Non-limiting

examples of such derivatives and fragments comprise hnRNP A1 *in vitro* mutagenized in the RRM1, RRM2 or the glycine-rich domain (see below).

5 Herein, hnRNP A1 is meant to designate the nucleic acid and/or the protein. It will be recognized by a person of ordinary skill whether the protein or nucleic acid fragment is intended.

In related aspects, the present invention features a pharmaceutical composition which include therapeutically effective amounts of modulators of telomere length or replication in accordance with the present invention and pharmaceutically acceptable buffers. In 10 one particular embodiment, these pharmaceutical compositions may include one or more of these inhibitors or agents and can be co-administered with other drugs. For example, AZT is commonly used for treatment of HIV, and may be co-administered with a telomere length reducing agent of the present invention.

15 In another related aspect, the invention features a method for extending the ability of a cell to replicate. In this method, a replication extending amount of an agent which is active to reduce loss of telomere length within the cell is provided during cell replication. As will be evident to those of ordinary skill in the art, this agent is similar to that 20 useful for treatment of a condition associated with an increased rate of proliferation of a cell. However, this method is useful for the treatment of individuals not suffering from any particular condition, but in which one or more cell types are limiting in that patient, and whose life can be extended by extending the ability of those cells to continue replication. 25 That is, the agent is added to delay the onset of cell senescence characterized by the inability of that cell to replicate further in an

individual. One example of such a group of cells includes lymphocytes present in patients suffering from Downs Syndrome (although treatment of such cells may also be useful in individuals not identified as suffering from any particular condition or disease, but simply recognize that one or 5 more cells, or collections of cells are becoming limiting in the life span of that individual).

It is notable that administration of such inhibitors or agents is not expected to be detrimental to any particular individual or animal. However, should gene therapy be used to introduce an agent of 10 the invention into any particular cell population, care should be taken to ensure that the activity of that agent is appropriately regulated, for example, by use of a promoter which can be regulated by the nutrition of the patient. Thus, for example, the promoter may only be activated when the patient eats a particular nutrient, and is otherwise inactive. In this way, 15 should the cell population become malignant, that individual may readily inactivate replication of the cell and cause it to become senescent simply by no longer eating that nutrient.

Another aspect of the present invention features a method for treatment of a condition associated with an elevated level of 20 telomerase activity and/or with longer and/or more stable telomeres within a cell. The method involves administering to that cell a therapeutically effective amount of an agent that reduces or destabilizes the length of the telomeres. The level of telomerase activity can be measured in accordance with the present invention or by any other existing method or 25 equivalent method. Examples of such conditions include neoplastic (cancerous) conditions, or conditions associated with the presence of

cells which are not normally present in that individual, such as protozoan parasites or opportunistic pathogens. Administration of such an agent can be achieved by any desired mean well known to those of ordinary skill in the art.

5 By "elevated level" of such activity, it is meant that the absolute level of telomerase activity in a particular cell is elevated compared to normal cells in that individual or compared to normal cells in other individuals not suffering from the same condition. The same principle applies to an elevated level or an elevated activity of A1 or UP1
10 on the length of telomeres.

In addition, the term "therapeutically effective amount" of an inhibitor or modulator is a well recognized phrase. The amount actually applied will be dependent upon the individual or animal to which treatment is to be applied, and will preferably be an optimized amount
15 such that an inhibitory effect is achieved without significant side-effects (to the extent that those can be avoided by use of the inhibitor). That is, if effective inhibition can be achieved with no side-effects with the inhibitor at a certain concentration, that concentration should be used as opposed to a higher concentration at which side-effects may become evident. If
20 side-effects are unavoidable, however, the minimum amount of inhibitor that is necessary to achieve the inhibition desired should be used. The terminology "effective amount" should be similarly understood while, in certain embodiments, the aspect of side effects and the like may or may not come into consideration.

25 By "inhibitor" is simply meant any reagent, drug or chemical which is able to inhibit the alternative splicing activity of A1 in

vivo or *in vitro*, sufficiently to affect telomere biogenesis. Such inhibitors can be readily identified using standard screening protocols in which A1 and the nucleic acid is placed in contact with a potential inhibitor and the level of splicing or the spliced products are measured or identified in the presence or absence of the inhibitor or in the presence of varying amounts thereof. In this way, not only can useful inhibitors (or stimulators) be identified, but the optimum level of such an inhibitor (or stimulator) can be determined *in vitro*. Once identified as a modulator *in vitro*, the agent can be tested *in vivo*. Numerous methods to test the *in vivo* effect of this modulator are known to the person skilled in the art to which this application pertains. In one particular embodiment, this agent is DMSO or derivatives thereof.

DMSO is a known chemical (see, for example, the Merck Index, 11th Edition, pages 513-514 and references therein). DMSO derivatives will be understood by a person skilled in the art to be an equivalent of C_2H_6OS , which retains its function as an inhibitor of hnRNP A1-dependent alternative splicing.

As used herein, the terms "molecule", "compound" or "ligand" are used interchangeably and broadly to refer to natural, synthetic or semi-synthetic molecules or compounds. The term "molecule" therefore denotes for example chemicals, macromolecules, cell or tissue extracts (from plants or animals) and the like. Non limiting examples of molecules include nucleic acid molecules, peptides, proteins (including dominant negative mutants; UP1 in certain conditions for example [Blanchette et al., 1999, *supra*]), antibodies, carbohydrates and pharmaceutical agents. The agents can be selected and screened by a

variety of means including random screening, rational selection and by rational design using for example protein or ligand modeling methods such as computer modeling, combinatorial library screening and the like. The terms "rationally selected" or "rationally designed" are meant to 5 define compounds which have been chosen based on the configuration of the interaction domains of the present invention. As will be understood by the person of ordinary skill, macromolecules having non-naturally occurring modifications are also within the scope of the term "molecule". For example, peptidomimetics, well known in the pharmaceutical industry 10 and generally referred to as peptide analogs can be generated by modeling as mentioned above. Similarly, in a preferred embodiment, the polypeptides of the present invention are modified to enhance their stability. It should be understood that in most cases this modification should not alter the biological activity of the interaction domain. The 15 molecules identified in accordance with the teachings of the present invention have a therapeutic value in diseases or conditions in which the physiology or homeostasis of the cell and/or tissue is compromised by a defect in telomere length control or modulation. Alternatively, the molecules identified in accordance with the teachings of the present 20 invention find utility in the development of more efficient modulators of telomerase length.

As used herein, agonists and antagonists of the A1-dependent splicing activity also include potentiators of known compounds with such agonist or antagonist properties. In one embodiment, agonists 25 can be detected by contacting the indicator cell with a compound or mixture thereof or library of molecules (e.g. combinatorial library) for a

fixed period time and determining a biological activity as described herein. Of course, antagonists can be similarly detected.

The therapeutic aspect of the invention is related to the now clear observation that the ability of a cell to remain immortal comprises the ability of that cell to maintain or increase the telomere length of chromosomes within that cell. Thus, therapeutic approaches for reducing the potential of a cell to remain immortal focuses on the inhibition of the activity of A1 on alternative splicing or on the level thereof, in those cells in which it is desirable to cause cell death. Example 5 of such cells, but not limited to, include cancerous cells, which are one example of somatic cells which show increased length or stability of telomeres, and have become immortal. The present invention now permits such cells to become mortal once more by a reduction in the size 10 or the stability of the telomeres. As such, inhibition can be achieved in a multitude of ways as, for example, by providing inhibitors, dominant 15 negative mutants, derivatives of these dominant negative mutants and the like.

The inhibitors may be used for treatment of cancers of any type non-limiting examples thereof, including solid tumors and 20 leukemias, carcinoma, histiocytic disorders, leukemia, histiocytosis malignant, Hodgkin's disease, immunoproliferative small, non-Hodgkin's lymphoma, plasmacytoma, reticuloendotheliosis, melanoma and the like, osteosarcoma, rhabdomyosarcoma, sarcoma, neoplasms, and for any 25 treatment or of all other conditions in which cells have become immortalized.

In other cases, it is important to slow the loss of telomere sequences, in particular, cells in association with certain diseases (although such treatment is not limited to this, it can be used in normal ageing and *ex vivo* treatments). For example, some diseases 5 display abnormal fast rate of proliferation of one or more particular groups of cells. One example of such a disease is AIDS, in which death is caused by the early senescence of CD4⁺ cells. It is important to note that such cells age, not because of abnormal loss of telomere sequences (although this may be a factor) but rather because the replicative rate of 10 the CD4⁺ cells is increased such that telomere attrition occurs at a greater rate than normal for that group of cells (Lundblad and Wright, 1996, *Cell* 87:369). Thus, the present invention provides means to stabilize the length of telomeres. The applicant therefore is providing therapeutic agents which can be used in the treatment of such diseases, and in 15 addition, the means of diagnostic procedures by which similar diseases can be detected so that appropriate therapeutic protocols can be devised and implemented.

Specifically, the loss of telomeres within any particular cell population can be reduced by providing thereto telomere length 20 stabilizing agents, telomere replication stimulators and the like, according to the present invention. These molecules can be provided within a cell in order to reduce telomere loss or to make that cell immortal. Equivalent of such molecules, or other molecules may be readily screened to determine those that will reduce loss of telomeres or stabilize the length 25 of same. Such screening may occur *in vitro*, and the therapeutic agents discovered by such screening utilized in the above method *in vivo*. It

should be understood that in some situations, *in vitro* assays such as splicing assays might be sufficient to assess the telomere length stabilizing activity of an agent. In other cases, the assessment of telomere length *per se* (as opposed to binding of an agent to the telomere) might 5 have to be ascertained in cultured cells for example. The skilled artisan will be able to determine which assay (which are not limited to the ones listed above) is sufficient to determine the effect of the tested agent on telomere length.

10 Nucleotide sequences are presented herein by single strand, in the 5' to 3' direction, from left to right, using the one letter nucleotide symbols as commonly used in the art and in accordance with the recommendations of the IUPAC-IUB Biochemical Nomenclature Commission.

15 Unless defined otherwise, the scientific and technological terms and nomenclature used herein have the same meaning as commonly understood by a person of ordinary skill to which this invention pertains. Generally, the procedures for cell cultures, infection, molecular biology methods and the like are common methods used in the art. Such standard techniques can be found in reference 20 manuals such as for example Sambrook et al. (1989, Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratories) and Ausubel et al. (1994, Current Protocols in Molecular Biology, Wiley, New York).

25 The present description refers to a number of routinely used recombinant DNA (rDNA) technology terms. Nevertheless, definitions of selected examples of such rDNA terms are provided for clarity and consistency.

As used herein, "nucleic acid molecule", refers to a polymer of nucleotides. Non-limiting examples thereof include DNA (i.e. genomic DNA, cDNA) and RNA molecules (i.e. mRNA). The nucleic acid molecule can be obtained by cloning techniques or synthesized. DNA 5 can be double-stranded or single-stranded (coding strand or non-coding strand [antisense]).

The term "recombinant DNA" as known in the art refers to a DNA molecule resulting from the joining of DNA segments. This is often referred to as genetic engineering.

10 The term "DNA segment", is used herein, to refer to a DNA molecule comprising a linear stretch or sequence of nucleotides. This sequence when read in accordance with the genetic code, can encode a linear stretch or sequence of amino acids which can be referred to as a polypeptide, protein, protein fragment and the like.

15 The terminology "amplification pair" refers herein to a pair of oligonucleotides (oligos) of the present invention, which are selected to be used together in amplifying a selected nucleic acid sequence by one of a number of types of amplification processes, preferably a polymerase chain reaction. Other types of amplification 20 processes include ligase chain reaction, strand displacement amplification, or nucleic acid sequence-based amplification, as explained in greater detail below. As commonly known in the art, the oligos are designed to bind to a complementary sequence under selected conditions.

25 The nucleic acid (i.e. DNA or RNA) for practicing the present invention may be obtained according to well known methods.

Oligonucleotide probes or primers of the present invention may be of any suitable length, depending on the particular assay format and the particular needs and targeted genomes employed. In general, the oligonucleotide probes or primers are at least 12 5 nucleotides in length, preferably between 15 and 24 molecules, and they may be adapted to be especially suited to a chosen nucleic acid amplification system. As commonly known in the art, the oligonucleotide probes and primers can be designed by taking into consideration the melting point of hybridization thereof with its targeted sequence (see 10 below and in Sambrook et al., 1989, Molecular Cloning - A Laboratory Manual, 2nd Edition, CSH Laboratories; Ausubel et al., 1989, in Current Protocols in Molecular Biology, John Wiley & Sons Inc., N.Y.).

The term "oligonucleotide" or "DNA" molecule or sequence refers to a molecule comprised of the deoxyribonucleotides 15 adenine (A), guanine (G), thymine (T) and/or cytosine (C), in a double-stranded form, and comprises or includes a "regulatory element" according to the present invention, as the term is defined herein. The term "oligonucleotide" or "DNA" can be found in linear DNA molecules or fragments, viruses, plasmids, vectors, chromosomes or synthetically 20 derived DNA. As used herein, particular double-stranded DNA sequences may be described according to the normal convention of giving only the sequence in the 5' to 3' direction.

"Nucleic acid hybridization" refers generally to the hybridization of two single-stranded nucleic acid molecules having 25 complementary base sequences, which under appropriate conditions will form a thermodynamically favored double-stranded structure. Examples

of hybridization conditions can be found in the two laboratory manuals referred above (Sambrook et al., 1989, *supra* and Ausubel et al., 1989, *supra*) and are commonly known in the art. In the case of a hybridization to a nitrocellulose filter, as for example in the well known Southern blotting procedure, a nitrocellulose filter can be incubated overnight at 5 65°C with a labeled probe in a solution containing 50% formamide, high salt (5 x SSC or 5 x SSPE), 5 x Denhardt's solution, 1% SDS, and 100 µg/ml denatured carrier DNA (i.e. salmon sperm DNA). The non-specifically binding probe can then be washed off the filter by several 10 washes in 0.2 x SSC/0.1% SDS at a temperature which is selected in view of the desired stringency: room temperature (low stringency), 42°C (moderate stringency) or 65°C (high stringency). The selected temperature is based on the melting temperature (T_m) of the DNA hybrid. Of course, RNA-DNA hybrids can also be formed and detected. In such 15 cases, the conditions of hybridization and washing can be adapted according to well known methods by the person of ordinary skill. Stringent conditions will be preferably used (Sambrook et al., 1989, *supra*).

Probes of the invention can be utilized with naturally occurring sugar-phosphate backbones as well as modified backbones 20 including phosphorothioates, dithionates, alkyl phosphonates and α-nucleotides and the like. Modified sugar-phosphate backbones are generally taught by Miller, 1988, Ann. Reports Med. Chem. 23:295 and Moran et al., 1987, Nucleic Acids Res., 14:5019. Probes of the invention can be constructed of either ribonucleic acid (RNA) or deoxyribonucleic 25 acid (DNA).

The types of detection methods in which probes can be used include Southern blots (DNA detection), dot or slot blots (DNA, RNA), and Northern blots (RNA detection). Although less preferred, labeled proteins could also be used to detect a particular nucleic acid 5 sequence to which it binds. Other detection methods include kits containing probes on a dipstick setup and the like.

Although the present invention is not specifically dependent on the use of a label for the detection of a particular nucleic acid sequence, such a label might be beneficial, by increasing the 10 sensitivity of the detection. Furthermore, it enables automation. Probes can be labeled according to numerous well known methods (Sambrook et al., 1989, *supra*). Non-limiting examples of labels include ^3H , ^{14}C , ^{32}P , and ^{35}S . Non-limiting examples of detectable markers include ligands, fluorophores, chemiluminescent agents, enzymes, and antibodies. Other 15 detectable markers for use with probes, which can enable an increase in sensitivity of the method of the invention, include biotin and radionucleotides. It will become evident to the person of ordinary skill that the choice of a particular label dictates the manner in which it is bound to the probe.

20 As commonly known, radioactive nucleotides can be incorporated into probes of the invention by several methods. Non-limiting examples thereof include kinasing the 5' ends of the probes using gamma ^{32}P ATP and polynucleotide kinase, using the Klenow fragment of Pol I of *E. coli* in the presence of radioactive dNTP (i.e. uniformly labeled DNA 25 probe using random oligonucleotide primers in low-melt gels), using the

SP6/T7 system to transcribe a DNA segment in the presence of one or more radioactive NTP, and the like.

As used herein, "oligonucleotides" or "oligos" define a molecule having two or more nucleotides (ribo or deoxyribonucleotides).

5 The size of the oligo will be dictated by the particular situation and ultimately on the particular use thereof and adapted accordingly by the person of ordinary skill. An oligonucleotide can be synthetised chemically or derived by cloning according to well known methods.

10 As used herein, a "primer" defines an oligonucleotide which is capable of annealing to a target sequence, thereby creating a double stranded region which can serve as an initiation point for DNA synthesis under suitable conditions.

15 Amplification of a selected, or target, nucleic acid sequence may be carried out by a number of suitable methods. See generally Kwoh et al., 1990, Am. Biotechnol. Lab. 8:14-25. Numerous amplification techniques have been described and can be readily adapted to suit particular needs of a person of ordinary skill. Non-limiting examples of amplification techniques include polymerase chain reaction (PCR), ligase chain reaction (LCR), strand displacement amplification (SDA),
20 transcription-based amplification, the Q β replicase system and NASBA (Kwoh et al., 1989, Proc. Natl. Acad. Sci. USA 86, 1173-1177; Lizardi et al., 1988, BioTechnology 6:1197-1202; Malek et al., 1994, Methods Mol. Biol., 28:253-260; and Sambrook et al., 1989, *supra*). Preferably, amplification will be carried out using PCR.

25 Polymerase chain reaction (PCR) is carried out in accordance with known techniques. See, e.g., U.S. Pat. Nos. 4,683,195;

4,683,202; 4,800,159; and 4,965,188 (the disclosures of all three U.S. Patent are incorporated herein by reference). In general, PCR involves, a treatment of a nucleic acid sample (e.g., in the presence of a heat stable DNA polymerase) under hybridizing conditions, with one 5 oligonucleotide primer for each strand of the specific sequence to be detected. An extension product of each primer which is synthesized is complementary to each of the two nucleic acid strands, with the primers sufficiently complementary to each strand of the specific sequence to hybridize therewith. The extension product synthesized from each primer 10 can also serve as a template for further synthesis of extension products using the same primers. Following a sufficient number of rounds of synthesis of extension products, the sample is analyzed to assess whether the sequence or sequences to be detected are present. Detection of the amplified sequence may be carried out by visualization 15 following EtBr staining of the DNA following gel electrophores, or using a detectable label in accordance with known techniques, and the like. For a review on PCR techniques (see PCR Protocols, A Guide to Methods and Amplifications, Michael et al. Eds, Acad. Press, 1990).

Ligase chain reaction (LCR) is carried out in accordance 20 with known techniques (Weiss, 1991, *Science* 254:1292). Adaptation of the protocol to meet the desired needs can be carried out by a person of ordinary skill. Strand displacement amplification (SDA) is also carried out in accordance with known techniques or adaptations thereof to meet the particular needs (Walker et al., 1992, *Proc. Natl. Acad. Sci. USA* 25 89:392-396; and *ibid.*, 1992, *Nucleic Acids Res.* 20:1691-1696).

As used herein, the term "gene" is well known in the art and relates to a nucleic acid sequence defining a single protein or polypeptide. A "structural gene" defines a DNA sequence which is transcribed into RNA and translated into a protein having a specific amino acid sequence thereby giving rise the a specific polypeptide or protein. It will be readily recognized by the person of ordinary skill, that the nucleic acid sequence of the present invention can be incorporated into anyone of numerous established kit formats which are well known in the art.

A "heterologous" (i.e. a heterologous gene) region of a DNA molecule is a subsegment segment of DNA within a larger segment that is not found in association therewith in nature. The term "heterologous" can be similarly used to define two polypeptidic segments not joined together in nature. Non-limiting examples of heterologous genes include reporter genes such as luciferase, chloramphenicol acetyl transferase, β -galactosidase, and the like which can be juxtaposed or joined to heterologous control regions or to heterologous polypeptides.

The term "vector" is commonly known in the art and defines a plasmid DNA, phage DNA, viral DNA and the like, which can serve as a DNA vehicle into which DNA of the present invention can be cloned. Numerous types of vectors exist and are well known in the art.

The term "expression" defines the process by which a gene is transcribed into mRNA (transcription), the mRNA is then being translated (translation) into one polypeptide (or protein) or more.

The terminology "expression vector" defines a vector or vehicle as described above but designed to enable the expression of an inserted sequence following transformation into a host. The cloned gene

(inserted sequence) is usually placed under the control of control element sequences such as promoter sequences. The placing of a cloned gene under such control sequences is often referred to as being operably linked to control elements or sequences.

5 Operably linked sequences may also include two segments that are transcribed onto the same RNA transcript. Thus, two sequences, such as a promoter and a "reporter sequence" are operably linked if transcription commencing in the promoter will produce an RNA transcript of the reporter sequence. In order to be "operably linked" it is
10 not necessary that two sequences be immediately adjacent to one another.

15 Expression control sequences will vary depending on whether the vector is designed to express the operably linked gene in a prokaryotic or eukaryotic host or both (shuttle vectors) and can additionally contain transcriptional elements such as enhancer elements, termination sequences, tissue-specificity elements, and/or translational initiation and termination sites.

20 Prokaryotic expressions are useful for the preparation of large quantities of the protein encoded by the DNA sequence of interest. This protein can be purified according to standard protocols that take advantage of the intrinsic properties thereof, such as size and charge (i.e. SDS gel electrophoresis, gel filtration, centrifugation, ion exchange chromatography...). In addition, the protein of interest can be purified via affinity chromatography using polyclonal or monoclonal
25 antibodies. The purified protein can be used for therapeutic applications.

The DNA construct can be a vector comprising a promoter that is operably linked to an oligonucleotide sequence of the present invention, which is in turn, operably linked to a heterologous gene, such as the gene for the luciferase reporter molecule. "Promoter" 5 refers to a DNA regulatory region capable of binding directly or indirectly to RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of the present invention, the promoter is bound at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases 10 or elements necessary to initiate transcription at levels detectable above background. Within the promoter will be found a transcription initiation site (conveniently defined by mapping with S1 nuclease), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not 15 always, contain "TATA" boxes and "CCAT" boxes. Prokaryotic promoters contain -10 and -35 consensus sequences which serve to initiate transcription and the transcript products contain Shine-Dalgarno sequences which serve as ribosome binding sequences during translation initiation.

20 As used herein, the designation "functional derivative" denotes, in the context of a functional derivative of a sequence whether an nucleic acid or amino acid sequence, a molecule that retains a biological activity (either functional or structural) that is substantially similar to that of the original sequence. This functional derivative or 25 equivalent may be a natural derivatives or may be prepared synthetically. Such derivatives include amino acid sequences having substitutions,

deletions, or additions of one or more amino acids, provided that the biological activity of the protein is conserved. The same applies to derivatives of nucleic acid sequences which can have substitutions, deletions, or additions of one or more nucleotides, provided that the 5 biological activity of the sequence is generally maintained. When relating to a protein sequence, the substituting amino acid as chemico-physical properties which are similar to that of the substituted amino acid. The similar chemico-physical properties include, similarities in charge, bulkiness, hydrophobicity, hydrophylicity and the like. The term 10 "functional derivatives" is intended to include "fragments", "segments", "variants", "analogs" or "chemical derivatives" of the subject matter of the present invention.

Thus, the term "variant" refers herein to a protein or nucleic acid molecule which is substantially similar in structure and 15 biological activity to the protein or nucleic acid of the present invention.

The functional derivatives of the present invention can be synthesized chemically or produced through recombinant DNA technology. all these methods are well known in the art.

As used herein, "chemical derivatives" is meant to cover 20 additional chemical moieties not normally part of the subject matter of the invention. Such moieties could affect the physico-chemical characteristic of the derivative (i.e. solubility, absorption, half life and the like, decrease of toxicity). Such moieties are exemplified in Remington's Pharmaceutical Sciences (1980). Methods of coupling these chemical-physical moieties 25 to a polypeptide are well known in the art.

The term "allele" defines an alternative form of a gene which occupies a given locus on a chromosome.

As commonly known, a "mutation" is a detectable change in the genetic material which can be transmitted to a daughter 5 cell. As well known, a mutation can be, for example, a detectable change in one or more deoxyribonucleotide. For example, nucleotides can be added, deleted, substituted for, inverted, or transposed to a new position. Spontaneous mutations and experimentally induced mutations exist. The 10 result of a mutations of nucleic acid molecule is a mutant nucleic acid molecule. A mutant polypeptide can be encoded from this mutant nucleic acid molecule.

As used herein, the term "purified" refers to a molecule having been separated from a cellular component. Thus, for example, a 15 "purified protein" has been purified to a level not found in nature. A "substantially pure" molecule is a molecule that is lacking in all other cellular components.

In certain embodiments, it might be beneficial to use fusion proteins comprising the protein of the present invention, a part thereof or a derivative thereof. Non limiting examples of such fusion 20 proteins include a hemagglutinin fusions and Gluthione-S-transferase (GST) fusions and Maltose binding protein (MBP) fusions. In certain embodiments, it might be beneficial to introduce a protease cleavage site between the two polypeptide sequences which have been fused. Such protease cleavage sites between two heterologously fused polypeptides 25 are well known in the art.

In certain embodiments, it might also be beneficial to fuse the protein of the present invention, a part thereof or a derivative thereof, to signal peptide sequences enabling a secretion of the fusion protein from the host cell. Signal peptides from diverse organisms are 5 well known in the art. Bacterial OmpA and yeast Suc2 are two non-limiting examples of proteins containing signal sequences. In certain embodiments, it might also be beneficial to introduce a linker (commonly known) between the interaction domain and the heterologous polypeptide portion. Such fusion protein find utility in the assays of the present 10 invention as well as for purification purposes, detection purposes and the like.

For certainty, the sequences and polypeptides useful to practice the invention include without being limited thereto mutants, homologs, subtypes, alleles and the like. It shall be understood that 15 generally, the sequences of the present invention should encode a functional (albeit defective) interaction domain. It will be clear to the person of ordinary skill that whether an interaction domain of the present invention, variant, derivative, or fragment thereof retains its function in binding to its partner can be readily determined by using the teachings 20 and assays of the present invention and the general teachings of the art.

As exemplified herein below, the protein of the present invention, a part thereof or a derivative thereof, can be modified, for example by *in vitro* mutagenesis, to dissect the structure-function relationship thereof and permit a better design and identification of 25 modulating compounds. However, some derivative or analogs having lost their biological function may still find utility, for example for raising

antibodies. These antibodies could be used for detection or purification purposes. In addition, these antibodies could also act as competitive or non-competitive inhibitors and be found to be modulators of protease activity.

5 The antibodies of the present invention include monoclonal and polyclonal antibodies, as well as fragments of these antibodies. The invention further includes single chain antibodies. Antibody fragments which contain the idiotype of the molecule can be generated by known techniques. For example, such fragments include
10 but are not limited to: the $F(ab')_2$ fragment; the Fab' fragments, Fab fragments, and Fv fragments.

Of special interest to the present invention are produced in humans, or are "humanized" (i.e. non-immunogenic in a human) by recombinant or other technology. Humanized antibodies can be
15 produced for example by replacing an immunogenic portion of an antibody with a corresponding, but non-immunogenic portion (i.e. chimeric antibodies) (Robinson et al., International Patent Publication PCT/US86/02269; Akira et al., European Patent Application 184,187; Taniguchi, European Patent Application 171,496. Reviews on humanized
20 chimeric antibodies include Morrison, 1985, *Science* 229: 1202-1207 and Oi et al., 1986, *BioTechniques* 4:214.

In general, techniques for preparing monoclonal antibodies and hybridomas are well known in the art (Campbell, 1984, *supra*; Harlow et al., 1988, *supra*; and St. Groth et al., 1980, *J. Immunol. Methods* 35: 1-21. In general, techniques for purifying monoclonal antibodies are also well known in the art (Campbell, 1984, *supra*; Harlow

et al., 1988, *supra*). Non-limiting examples of monoclonal antibody purification methods include ammonium sulfate precipitation, ion exchange chromatography and HPLC. Monoclonal antibodies can also be produced by bioreactor such as the hollow fiber cell culture system 5 described in (the Unisyn instruction manuel). For example, using this hollow fiber membrane having a molecular weight cut off of 35,000, 1 x 10⁸ cells of hybridoma are introduced into the bioreactor. The hybridoma can be grown in PFHM-11 media (GIBCO, BRL) with PEN/STREP (GIBCO/BRL). In certain embodiments of the present invention, it might 10 be advantageous to provide the above-described antibodies as detectably labeled.

From the specification and appended claims, the term therapeutic agent should be taken in a broad sense so as to also include a combination of at least two such therapeutic agents. Further, the DNA 15 segments or proteins according to the present invention can be introduced into individuals in a number of ways. For example, erythropoietic cells can be isolated from the afflicted individual, transformed with a DNA construct according to the invention and reintroduced to the afflicted individual in a number of ways, including 20 intravenous injection. Alternatively, the DNA construct can be administered directly to the afflicted individual, for example, by injection in the bone marrow. The DNA construct can also be delivered through a vehicle such as a liposome, which can be designed to be targeted to a specific cell type, and engineered to be administered through different 25 routes.

For administration to humans, the prescribing medical professional will ultimately determine the appropriate form and dosage for a given patient, and this can be expected to vary according to the chosen therapeutic regimen (i.e. DNA construct, protein, cells), the response and condition of the patient as well as the severity of the disease.

Composition within the scope of the present invention should contain the active agent (i.e. fusion protein, nucleic acid, and molecule) in an amount effective to achieve the desired therapeutic effect while avoiding adverse side effects. Typically, the nucleic acids in accordance with the present invention can be administered to mammals (i.e. humans) in doses ranging from 0.005 to 1 mg per kg of body weight per day of the mammal which is treated. Pharmaceutically acceptable preparations and salts of the active agent are within the scope of the present invention and are well known in the art (Remington's Pharmaceutical Science, 16th Ed., Mack Ed.). For the administration of polypeptides, antagonists, agonists and the like, the amount administered should be chosen so as to avoid adverse side effects. The dosage will be adapted by the clinician in accordance with conventional factors such as the extent of the disease and different parameters from the patient. Typically, 0.001 to 50 mg/kg/day will be administered to the mammal.

Other objects features and advantages of the present invention will become apparent upon reading of the following non-restrictive description of the preferred embodiments thereof given by way of example only with reference to the accompanying drawings and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Having thus generally described the invention, reference will now be made to the accompanying drawings, showing by way of illustration a preferred embodiment thereof, and in which:

5 Figure 1 shows that DMSO affects 5' splice site selection but not generic splicing;

Figure 2 shows that DMSO affects 5' splice site selection in an hnRNP A1-dependent manner;

10 Figure 3 shows that DMSO affects 3' splice site selection;

Figure 4 shows that the effect of DMSO is reversible by adding more A1;

Figure 5 shows that DMSO affects NCAM alternative splicing *in vivo* and *in vitro*;

15 Figure 6 shows that DMSO affects hnRNP A1 alternative splicing *in vivo*;

Figure 7 shows DMSO does not affect hnRNP A1 binding; and

20 Figure 8 shows the mechanism of action of hnRNP A1 in alternative splicing and the block by DMSO.

Other objects, advantages and features of the present invention will become more apparent upon reading of the following non-restrictive description of preferred embodiments with reference to the accompanying drawing which is exemplary and should not be interpreted 25 as limiting the scope of the present invention.

DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention therefore provides the means to modulate telomere biogenesis by modulating alternative splicing of A1. In addition, it provides the means to modulate the alternative splicing of other pre-mRNAs and methods of identifying agents which modulate same.

EXAMPLE 1

10 **Use of DMSO to control the function of hnRNP A1 in alternative RNA splicing and telomere biogenesis**

Control of alternative splicing

15 DMSO is used to control the alternative splicing profile of pre-mRNAs when hnRNP A1 is involved in the modulation of splicing. Because the understanding of the mechanism of alternative splicing is still limited, few pre-mRNAs are known to use hnRNP A1 to control their alternative splicing profile. However, this can now be readily assessed by treating cells with DMSO and monitor the splicing profile of any genes by well-known assays like RT-PCR. Confirmation of the role of A1 can be done by performing in vitro splicing assays in DMSO-containing extracts.

20 Upon the completion (and partial completion) of the human genome project, the sequenced gene will provide the predicted splice consensus sequences, and ultimately the predicted alternative splicing profile of many genes. The present invention will enable a rapid and simple means of assessing the contribution of A1 in modulating the alternative splicing profile of as many genes as desired and to confirm the existence of the predicted alternative splicing events.

In certain conditions, it may be suitable to modulate the alternative splicing profile in order to generate protein isoforms with different activities. Depending on the target pre-mRNA and the type of cells where this gene is expressed, modulating alternative splicing may 5 have a considerable impact on cell growth and other cell properties. For example, certain types of cancer may produce protein isoforms that are important for continued cell growth. Thus, the use of an agent which could modify the alternative splicing profile giving rise to at least one different (or changing the level of) protein isoform could have a major 10 impact on cell growth and the control thereof. In a particular embodiment, DMSO treatment may shift the profile of alternative splicing and promote the production of a protein isoform that prevent cell growth or promote cell death. In other conditions affecting another cell type expressing different subsets of genes, the reverse may be true, i.e., DMSO may abrogate 15 apoptosis or permit cell division. The determination of the effect of DMSO treatment on particular genes and cells will require knowledge of all the genes expressed in a /given cell type and the alternative splicing profiles of these genes. While such information is already available for a number of genes, more will be available soon thanks to the DNA chips technology 20 (e.g. microarrays).

Thus, DMSO could be used to treat a variety of conditions including cancer and any diseases where the expression of certain spliced isoforms makes an important contribution to the disease (e.g. physiologically relevant). In such a case a topical application of 25 DMSO may promote cell death by altering the alternative splicing profile

of a target pre-mRNA that encodes for example a transcription control factor, a membrane receptor, and other cell growth modulators.

In one particular embodiment of the present invention, a variety of DMSO derivatives can be produced chemically and tested in 5 an assay of the present invention. These may be more potent than DMSO to modulate alternative splicing. Although DMSO can be viewed as a relatively safe product, derivatives may have additional advantages when topical application is considered.

Control of telomere biogenesis

10 Since hnRNP A1 controls telomere biogenesis, inactivation of the interaction properties of A1 with DMSO can be used to promote telomere shortening. A topical application to a tumor may help achieve telomere erosion in cancer cells and thus promote cell death. DMSO is readily adsorbed by cells and tissues and has been used in the 15 treatment of arthritis. Thus, surface tumors will be most easily treated by a DMSO application.

20 Since the present invention also provides agents which could promote telomere lengthening, the present invention broadly concerns agents which promote telomere length modulation and methods of identifying same.

While it is currently unknown how many genes will use hnRNP A1 to control alternative splicing, given that A1 is very highly expressed in actively growing cells, it is expected that many genes will use A1 to modulate their alternative splicing profile.

25 Although the present invention has been described hereinabove by way of preferred embodiments thereof, it can be modified,

without departing from the spirit and nature of the subject invention as defined in the appended claims.

WHAT IS CLAIMED IS:

1. A method to modulate telomere biogenesis *in vitro* comprising a use of an effective amount of an agent capable of inhibiting 5 hnRNP A1 splicing.
2. The method of claim 1, wherein said agent is DMSO.
3. A method to modulate alternative splicing *in vivo* 10 comprising a use of an effective amount of DMSO.
4. The method according to claim 1, 2 or 3, wherein said alternative splicing is hnRNP A1-dependent.

15 5. A method to modulate telomere biogenesis comprising a use of an effective amount of DMSO.

6. A method to evaluate an involvement of hnRNP A1 in alternative splicing of a given pre-mRNA comprising an incubation of 20 a cell extract containing at least one of said pre-mRNA to be tested and a monitoring of one of a splicing profile of said pre-mRNA and of a level of splicing of said pre-mRNA.
7. A method of identifying agents which modulate 25 telomere biogenesis comprising an incubation of a pre-mRNA in a splicing

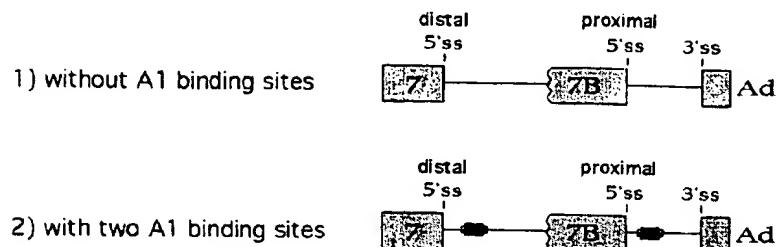
extract, wherein a change in splicing of said pre-mRNA can be detected and/or measured, and comparing qualitatively or quantitatively said splicing of said pre-mRNA in a presence of an agent (or a library thereof) and in an absence thereof (or library thereof), wherein an agent which 5 can modulate splicing and/or telomere biogenesis is identified when qualitative or quantitative difference in spliced products or splicing intermediates is measurably different in said presence of said agent as compared to in the absence thereof.

10

8. The method according to one of claims 1 to 7, wherein a DMSO derivative is used.

Figure 1 shows that DMSO affects 5' splice site selection but not generic splicing

A) Pre-mRNAs used (exon 7 and partial exon 7B of hnRNP A1 and terminal exon of adenovirus)



B) *In vitro* splicing with increasing amounts of DMSO (0.75, 1.5 and 2.2 %)

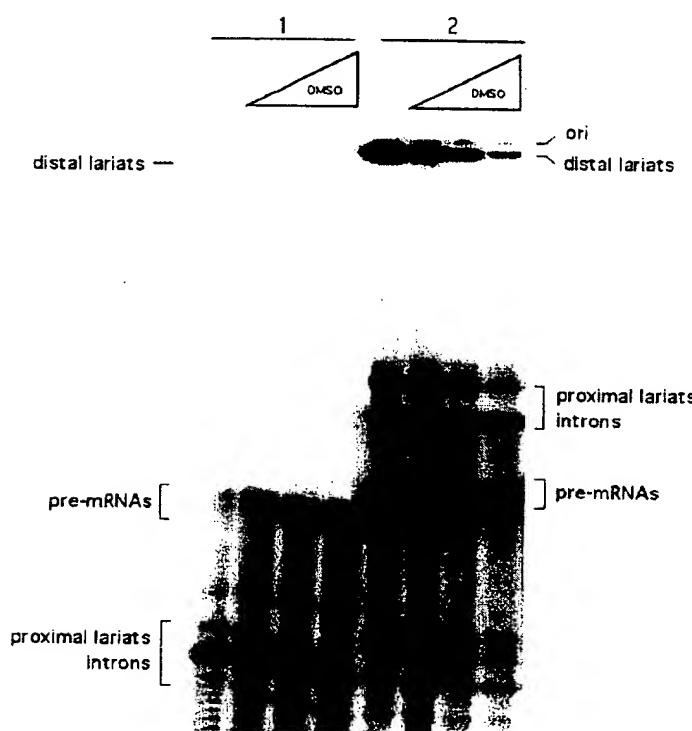
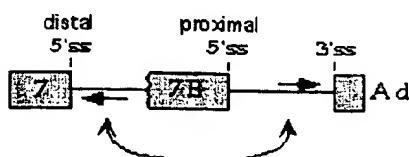


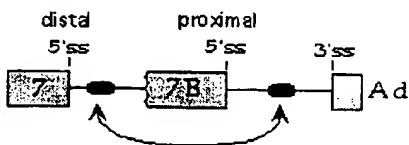
Figure 2 shows that DMSO affects 5' splice site selection in an hnRNP A1-dependent manner

A) Pre-mRNAs used (exons 7 and 7B of hnRNP A1 and adeno)

1) contains complementary sequences (20 nt each), which form a secondary structure



2) contains two A1 binding sites



B) *In vitro* splicing with increasing amounts of DMSO, using a nuclear extract with (NE) or without (NEΔA1) hnRNP A1

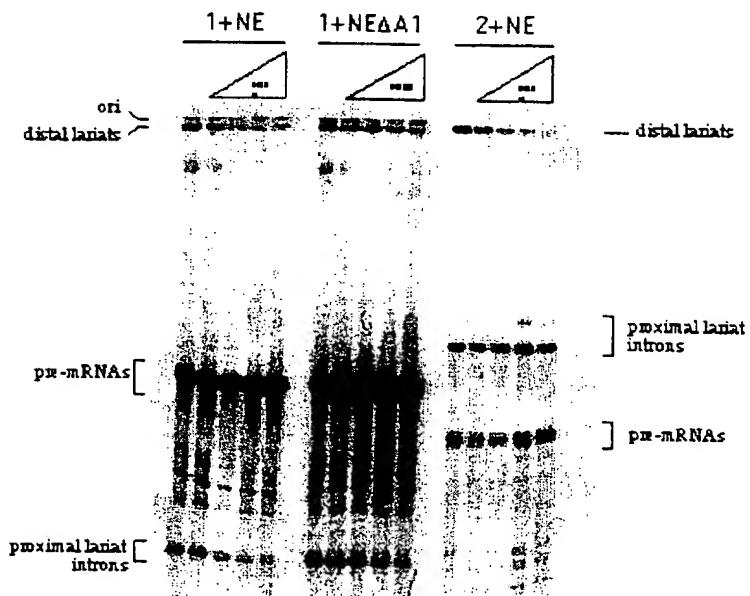
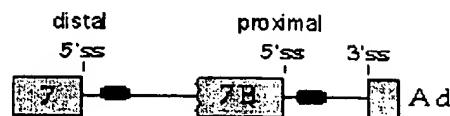


Figure 4 shows that the effect of DMSO is reversible by adding more A1

A) Pre-mRNA used (exons 7 and 7B of hnRNP A1 and adeno, two A1 binding sites)



B) *In vitro* splicing with increasing amounts of recombinant A1 protein

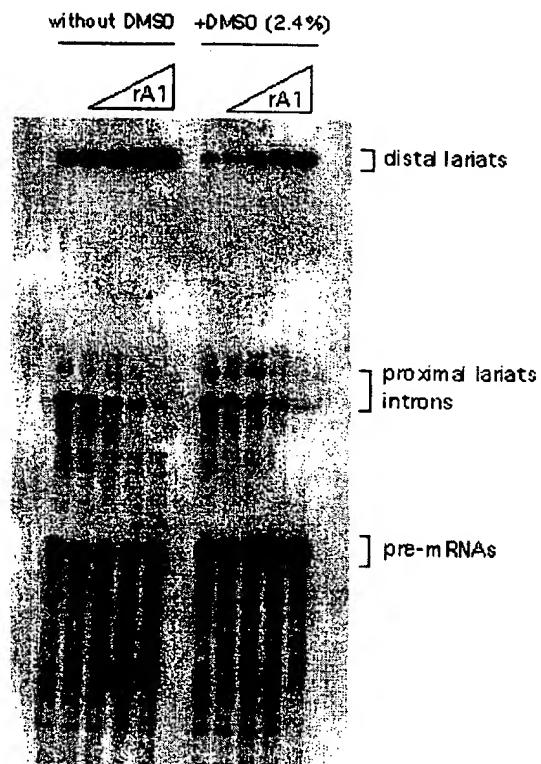
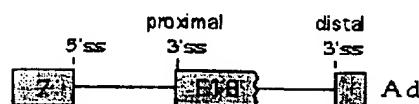
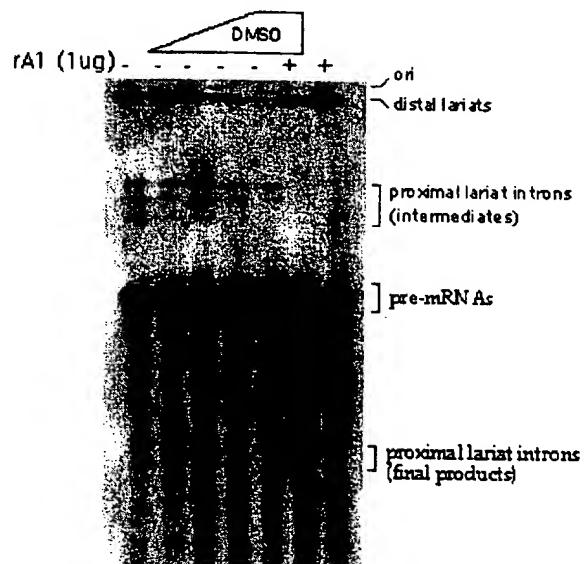


Figure 5 shows that DMSO affects NCAM alternative splicing *in vitro* and *in vivo*

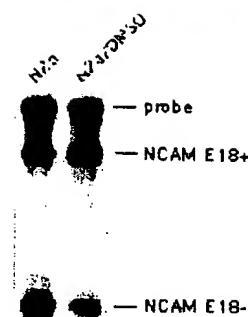
A) Pre-mRNA used (exon 7 of hnRNP A1, partial exon E18 of NCAM and adeno)



B) *In vitro* splicing with increasing amounts of DMSO

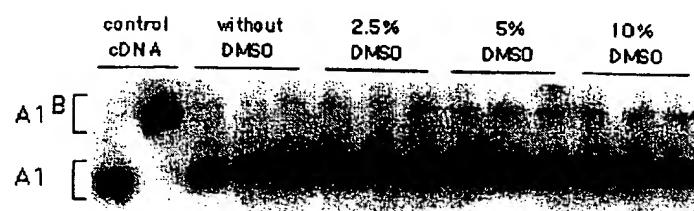


C) RNAse T1 protection assay



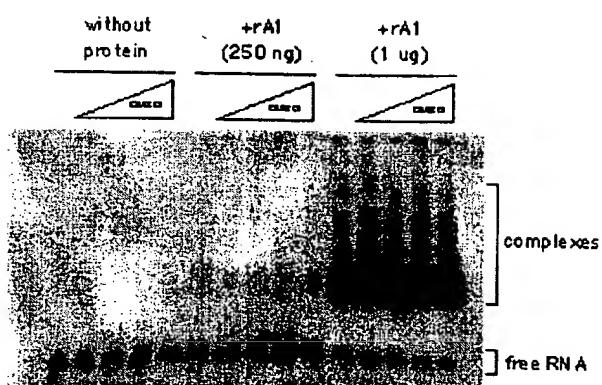
The N2a cells were treated (2%) or not with DMSO and the NCAM mRNAs were assayed in a RNAse T1 protection assay using a labelled RNA probe.

Figure 6 shows that DMSO affects hnRNP A1 alternative splicing *in vivo*



HeLa cells were treated with different amounts of DMSO (triplicate) for 5 hours and the mRNAs were amplified by RT-PCR and separated on a 5% polyacrylamide gel.

Figure 7 shows that DMSO does not affect A1 binding



concentrations of DMSO used: 0.8, 1.6, 2.4 and 3.2 %

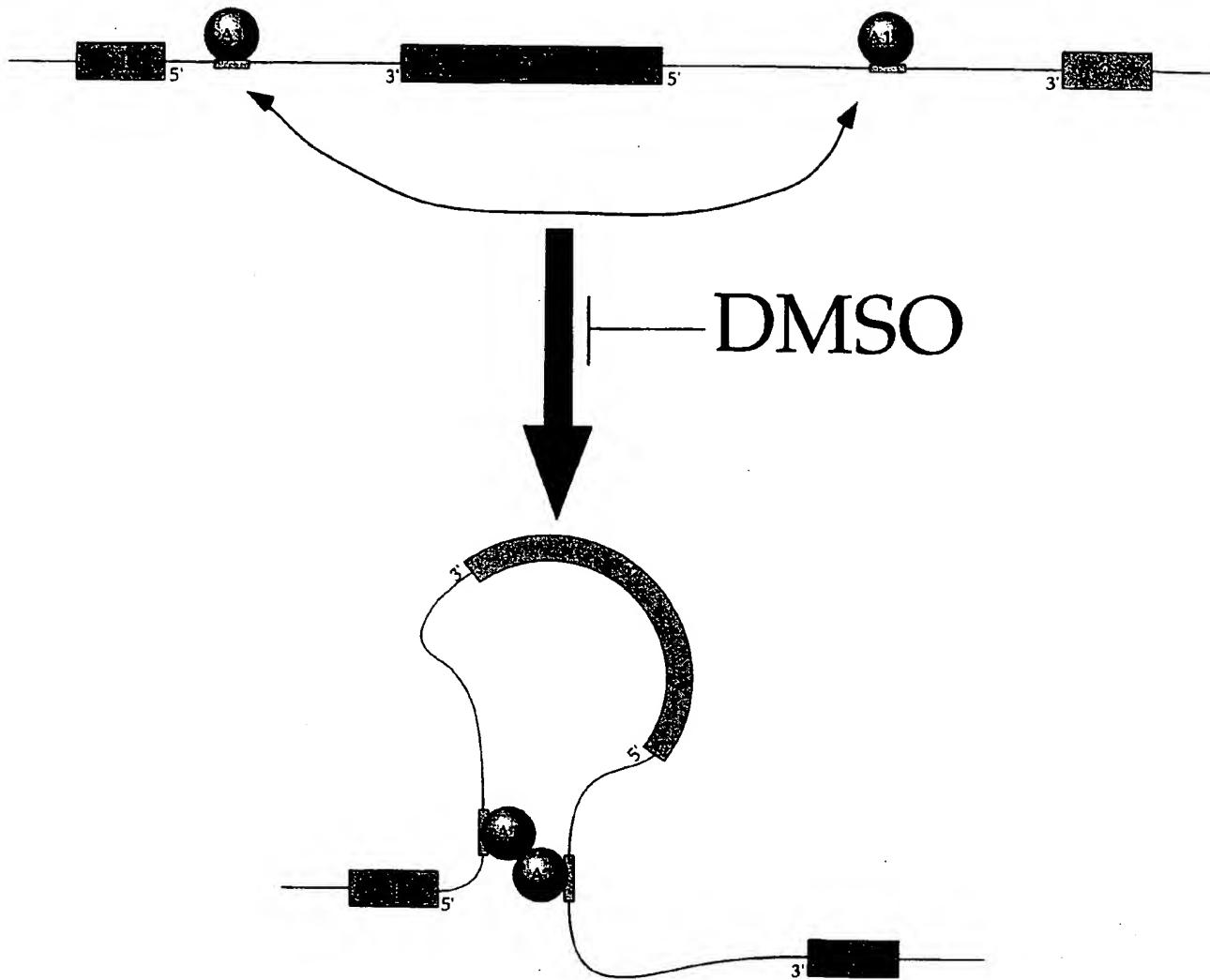


Figure 8. A1 proteins bound to high-affinity binding sites interact with one another. This interaction changes the conformation of the pre-mRNA and increases the frequency of splice site pairing between the 5' splice site of exon 1 and the 3' splice site of exon 3. DMSO does not prevent A1 binding but inhibits the use of the distal pair of splice sites.